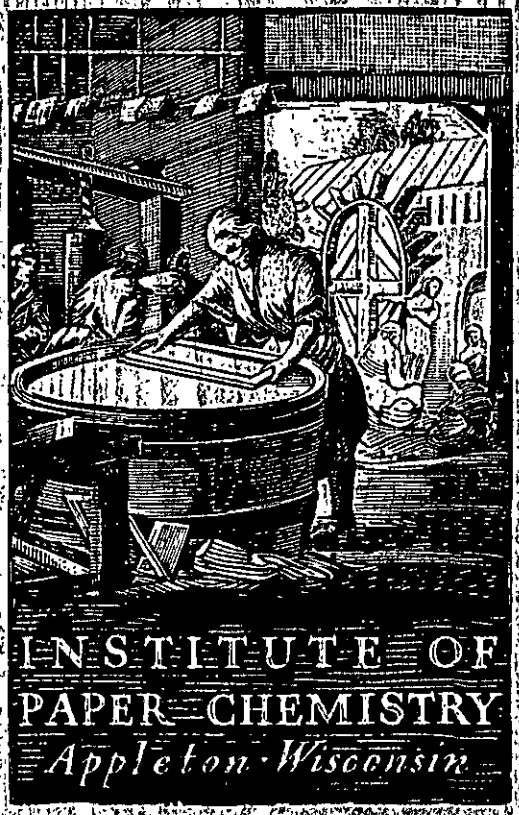

VEGETATIVE PROPAGATION OF ASPEN FROM CALLUS TISSUE

Project 2351

Report Eleven

The

Final Report

to

PIONEERING RESEARCH COMMITTEE

April 24, 1972

THE INSTITUTE OF PAPER CHEMISTRY

Appleton, Wisconsin

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SUMMARY

This report terminates Project 2351. For the past ten years, under two principal investigators, the work on aspen tissue culture finally culminated in the first reproduction of a tree species from callus cultures. Triploid quaking aspen, tetraploid European aspen, and a diploid hybrid between quaking aspen and white poplar have been reproduced by this method, and unrooted shoots have been produced for 5-6 other aspen species. This report contains additional studies to improve the nutrient medium, to increase the growth of firm white callus. Shoot initiation tests are also reported, using callus grown on the various new media. Apparently, shoots can be initiated equally well on seven-month-old callus from either of two clones of triploid quaking aspen, from tissue grown on medium containing either IAA or 2,4-D as the auxin growth hormone. Trees were produced from two-year-old callus, and both the rate of growth and shoot-initiation potential have declined during the past three years. For both growth and shoots, glycine inhibits and hydroxyproline stimulates. The first green callus of triploid quaking aspen was grown in the light, after one month in liquid medium in the dark followed by one month in liquid in the light. This project proved that trees can be reproduced from callus tissue, and now we are ready to invite support for a new proposal to reproduce trees from single cells.

INTRODUCTION

The Pioneering Research Program was started in 1959, for the purpose of supporting studies of basic inquiry having an ultimate significance to the pulp and paper industry. Twenty-four member companies of The Institute of Paper Chemistry underwrote this program and, under the direction of Chairman Dr. Roy P. Whitney, fifteen projects were supported among thirteen institutions in five countries. Each study considered some aspect of the biochemical, molecular, structural, or mechanical nature of fibrous materials. In October, 1964, the Five-Year Technical Meeting of The Pioneering Research Program was held in Appleton, Wisconsin, and reports of each project were presented to representatives of the sponsoring companies.

In 1962, a three-year grant was awarded to Dr. Martin C. Mathes at The Institute of Paper Chemistry, to study DNA recombination and also to produce callus cultures from pollen. After preliminary experiments, the direction of the project was changed with the approval of The Pioneering Research Committee, to investigate the possible use of tissue culture in studies of the physiology and vegetative propagation of aspen species. Mathes initiated callus cultures from six species of foreign and domestic aspen (1), including the rare triploid form of normally diploid quaking aspen. About one-dozen clonal groups of natural triploids have been found in the Lake States region, and contain three sets of chromosomes in each newly-formed cell instead of the normal two sets. This extra set of hereditary units results in 30-40% faster growth and superior pulping properties over the diploid form.

Mathes developed a satisfactory nutrient agar medium for triploid quaking aspen (Populus tremuloides Michx.) by adapting popular White's medium and adding

15% deproteinized coconut milk. With callus initiated in 1961 (2), Mathes studied antimicrobial exudants (3) and grew roots and shoots from the same piece of callus (4). However, the roots were not connected to the shoots, so complete plants were not formed. Several years later, however, he was able to excise shoots and root them in medium (personal communication). In 1964, Mathes moved to The College of William and Mary in Williamsburg, Virginia. Callus that he took with him has since been used to study bacterial inhibition (5) and chloroplast ultrastructure (6). In addition, data that he collected at the Institute was compared with later studies made by Kremers, to show that callus tissue was chemically similar to xylem-initial cells normally produced in tree stems of aspen (7).

One week before Mathes left, Dr. Lawson L. Winton arrived and was appointed as the principal investigator for Project 2351. A second grant was awarded to the Project in 1965, and a third in 1967. In 1971, after all other research projects had been terminated, the uncommitted funds for the program were transferred to Project 2351. This money is now nearly exhausted, and the Project is expected to terminate in April, 1972. The closing of this ten-year project also brings to an end the thirteen-year Pioneering Research Program.

Table I compares several aspects of the Project under the two principal investigators Mathes and Winton. Mathes actually initiated his first callus in December, 1961, under another project, and so technically spent only two years on Project 2351. Winton spent four times this duration but almost eight times as much money. Mathes developed the sterilization technique which is still used, and is coauthor with Winton for a recently-submitted invited book chapter on the initiation of aspen callus. This book is scheduled for publication by Academic Press in 1973, and will be titled "Methods and Applications of Tissue Culture," covering both plant and animal techniques.

TABLE I

COMPARISONS OF PROJECT 2351 UNDER THE TWO PRINCIPAL INVESTIGATORS

Principal Investigator	Mathes	Winton
Duration	1962-4	1964-72
Years	2	8
Group Coordinator	Genetics & Physiology Dr. Dean Einspahr	
Section Chairman	Biology Dr. Willis Van Horn	
Division 1969		Natural Materials & Systems
Director		John Swanson
Laboratory Assistants	Mrs. Dorothy Olson Mrs. Marianne Harder[a]	
1966		Mrs. Susan Lebergen
1968		Mrs. Shirley Verhagen
Research Report Numbers	1-4	5-11
Research Notebooks	2032 2146 2156	2156 2405 2650 2801
Publications	8	16
Cost	$30,000	$231,000

[a]Mrs. Harder is now Assistant to the Group Coordinator, Dr. Einspahr.

Aspen species were first cultured at The Institute of Paper Chemistry in 1957 by Dr. Johannes van Buijtenen, now with the Texas Forest Service. However, these cultures could not be maintained, due to insufficient knowledge of the nutrient requirements. Mathes initiated callus in 1961, on a nutrient medium containing incompletely-defined coconut milk. Winton spent one year trying to develop a completely-defined medium, but was unsuccessful until a copy of Wolter's

thesis (8) was obtained. Winton modified the Wolter and Skoog (9) medium, on which Dr. Karl Wolter obtained stunted shoots and roots from aspen callus (10). Callus initiated by Winton in 1966-68 (11-13) was grown in liquid shake cultures as firm yellow callus, then was transferred to agar medium after different periods of time, and its ability to initiate roots was tested (14).

After one year on agar Medium 1 (14), firm white callus was growing slowly and uniformly, but no shoots had been initiated. Karl Wolter changed his research objectives at the Forest Products Laboratory in Madison, and met with Winton on three occasions during the fall of 1967. Wolter's levels of growth hormones were used by Winton, who produced shoots within two weeks (15). Some callus was transferred to supplemented media for another year, then very vigorous shoots were produced on shoot-initiation medium. Seven shoots grew their own roots while still attached to the callus, and four survived to grow into the first trees reproduced from callus tissue (16). The first tree grew its shoot on October 14 and its root November 1, 1968, and was planted on The Institute of Paper Chemistry's lawn July 11, 1969. New leaves started to grow on Moon Day, July 20, when the first man stepped onto the moon. The second tree will be used April 18, 1972, to commemorate the one-hundreth anniversary of Arbor Day at the National Arboretum in Washington, D.C. The third tree will be planted in Madison, in Capital Park; and the fourth tree is scheduled for planting in Minnesota, at the memorial to the late Professor Scott S. Pauley.

A second aspen species was reproduced from callus (17) originating from a tetraploid European aspen (P. tremula L.). A hybrid between diploid quaking aspen and white poplar (P. alba L.) has also been reproduced. However, shoots initiated from callus of many other species and hybrids of aspen did not grow roots and thus were not reproduced from callus. But the fact has been established, that tree

species in one genus have been reproduced from undifferentiated callus tissue. An extensive study was then made to excise shoots from callus and root them in different media. The best results gave 60% rooting, but tests made the following year were disappointing because shoots were not vigorous enough to root. Apparently, trees can be produced from callus during the second or third year from callus initiation, but shoot initiation declines with age of the callus. Consequently, large-scale shoot initiation from callus tissue does not appear to be a promising commercial method of vegetative propagation for aspen.

The first of the four trees reproduced from callus tissue is shown in Fig. 1 at the end of its third growing season. In 1969, Tree I only grew a few inches, giving it a height of about four feet. In 1970, another 1.5 feet was added; and it grew about one foot in 1971, for a total of 6.3 feet. This is very slow growth for quaking aspen, particularly the triploid. The dry season following planting, as well as rodent damage to the stem base in 1969-71 probably are responsible for the slow growth. One interesting sidelight, and probably the result of the weakening of the tree, is that Tree I put out seven male buds in 1971, and now has 40 male buds that will open this spring in 1972. Normally, quaking aspen does not flower for 8-9 years in a nursery and much later in the field.

Many herbaceous angiosperm plants, as well as several monocotyledonous (grass) plants have been reproduced from single cells. This now appears to be the most promising method of mass producing genetically-identical trees. A proposal is being submitted to funding organizations, requesting financial support for a new attack on reproducing trees from single cells in liquid suspensions. The cell suspension method is being investigated intensively for food plants, in hope of easing the world food shortage. Likewise, we believe that adapting this method to trees will help provide a continuous supply of wood.

Figure 1. Tree I, One of Four Triploid Quaking Aspen Reproduced from Callus Tissue in 1968-69. This Tree was 6.3 Feet Tall in September, 1971 at the End of the Third Growing Season. Black Wound Dressing Covers the Rodent Damage on the Stem. Dr. Winton is Standing Behind Tree I. The Inset Shows Three of the 1971 Male Buds

At this point in time, we still cannot predict the impact of our research with callus tissue on the future of a continuous wood supply. Already we can see the forests being pushed back onto less productive lands and their place taken by urbanization or recreational centers. In the far-distant future, if cellulose is

used at all, it may be synthesized in huge culture vats, thus eliminating the need for large forests. However, we are convinced that trees will continue to provide the cheapest supply of cellulose fibers in most countries for a long time, so we must develop new methods to grow high-quality trees faster on less land.

The reproduction of aspen from callus tissue is now very inefficient, and it will take considerable work to adapt this method to other tree species. If the callus method can be made more efficient, perhaps it can be used for limited commercial propagation of superior trees in the forest. These genetically identical trees would then be used in breeding and selection programs to develop parent trees. The superior parent trees would then produce seeds and seedlings, which would eventually be used to replant our diminishing forest lands.

However, as already stated, reproduction from single cells in suspension now appears to hold the greatest promise for mass producing genetically identical trees. So, the greatest contribution from Project 2351 may be the foundation of knowledge upon which to draw in learning more about cell cultures.

STOCK CULTURES OF TRIPLOID QUAKING ASPEN T-2-56

The data presented this year are from additional growth studies to improve the nutrient medium for aspen callus. When not otherwise qualified, aspen callus will mean that initiated from rooted root-sprouts of the triploid quaking aspen clone T-2-56, on January 23, 1967. In March, 1972, the callus had been subcultured (old callus cut into small cubes 3 mm. along each side) every 3-5 weeks for 61 passages. Since December, 1968, some callus has been subcultured on supplemented media for the past 48 passages. These media are documented in several past reports, as well as in last year's Report Ten (page 6). Our basic aspen Medium 1 was compared to the original Wolter and Skoog (9) medium, and to the older, but more universally accepted medium devised by Murashige and Skoog (18).

In the same Report Ten, 22 amino acids, one vitamin (folic acid) and four nucleotides plus thymine are listed as supplements to Medium 1 to give Medium 100. When the amino acid tryptophan is omitted, the medium is designated as 104. Many of our past reports have shown the general inhibiting effect of tryptophan on callus growth, as well as subsequent shoot initiation when the callus is placed on shoot-initiation Medium BAP-.05 or BAP-.10. The numbers represent the concentration in milligrams per liter of the cytokinin N^6-benzylaminopurine (BAP). The general composition of our four main media are listed in Table II.

TABLE II

PRINCIPAL ASPEN MEDIA

Medium	Use	General Composition	Mg./Liter		
			Auxin	Cytokinin	
			2,4-D	Kinetin	BAP
1	Slow firm callus	Modified Wolter & Skoog	0.04	1	--
100	Fast firm callus	W & S plus supplements	0.04	1	--
104	Fastest growth	W & S plus supplements, no tryptophan	0.04	1	--
BAP	Shoot initiation	Modified Wolter & Skoog	--	--	0.05-0.1

TRYPTOPHAN AND HYDROXYPROLINE

In Report Ten, we showed fluctuations in growth during a two-year period of callus grown on Media 1, 100, or 104 for consecutive four-week passages. During periods of rapid growth, callus on Medium 104 was generally the fastest growing, followed by callus on Medium 100, then Medium 1. Occasionally, Callus 100 outgrew Callus 104, and in periods of slow growth Callus 1 sometimes was intermediate between 100 and 104. We also showed that callus subcultured consecutively at five- or six-week passages usually gave larger callus pieces than at four weeks. Occasionally, though, callus consistently subcultured for six weeks seemed to decline in quality over several passages.

Hydroxyproline has been linked with cell-wall growth by workers such as Lamport (19). We grew callus on the three stock Media 1, 100, and 104, as well as on 104H with the hydroxyproline omitted. At the end of the regular four-week passage, wet-weight increments were determined and only callus on Medium 100 was significantly heavier than on all other three media (Fig. 2). In order to determine the effects of hydroxyproline and tryptophan on older callus, we left the callus pieces on the original three dishes and weighed each of the fifteen pieces each week for an additional six weeks.

Figure 2 shows the cumulative weekly increments on the three media. In the fifth week, Callus 100 immediately dropped to the second position in growth after 104, followed by 104H and 1. This order was maintained for the rest of the growth trial. Callus 1 significantly slowed in growth below the other three, giving a maximum wet weight increment of about one-half a gram. The vertical lines in Fig. 2 cover means not different from one another at the 5% level, according to Duncan's Multiple Test. Only at 6 and 7 weeks after subculture was

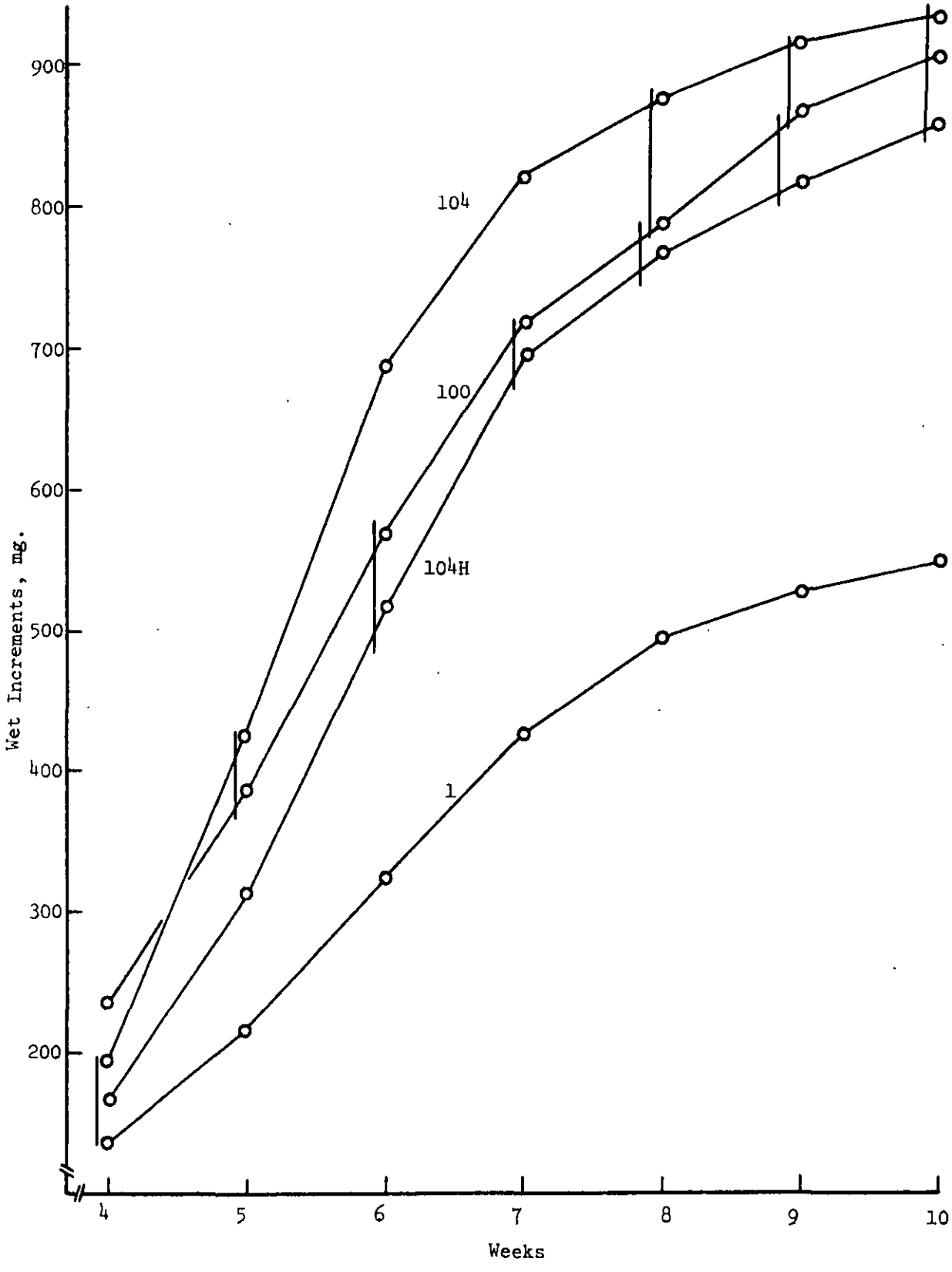

Figure 2. Wet-Weight Increment Growth of Callus on Media 1, 100, and 104, as well as on Medium 104H Made Without Hydroxyproline. Vertical Lines Join Means not Significantly Different at the 5% Level

the wet weight of Callus 104 significantly greater than 100 or 104H. However, these weeks represent the end of the rapid log-phase of growth, and the heavier callus on Medium 104 shows that hydroxyproline, but not tryptophan, was required for the fastest growth of firm white callus during those two weeks. For the rest of the time, neither tryptophan nor hydroxyproline seemed necessary for fast growth. However, all supplemented media gave faster growth than on basic Medium 1, as can be seen in Fig. 3. One can easily see the slower growth on Medium 1, but the faster growth among the other three media was not significantly different. However, the callus on 104H (Fig. 3D) appears to have more darker areas than on 104 (Fig. 3C), indicating that perhaps hydroxyproline helps to maintain high-callus quality. Callus 100 (Fig. 3B) shows much necrosis, and differs from the best callus on 104 (Fig. 3C) only by containing tryptophan. Tryptophan is a precursor to natural auxin IAA, and here shows a probable inhibiting effect on callus growth over long periods of time. It may also be that the tryptophan aids in the deposition of lignin or other organic compounds in the wall, which are not deposited when the auxin 2,4-D is present without tryptophan.

Figure 3. Aspen Callus Grown on Medium 1 (Fig. 3A), Medium 100 (Fig. 3B), Medium 104 (Fig. 3C), and Medium 104H (Fig. 3D) for Six Weeks in the Dark at 27°C.

GLYCINE AND HYDROXYPROLINE

Early in 1965, we determined that glycine inhibits the growth of aspen callus, and so Medium 1 does not contain this amino acid. However, when the five different groups of supplements were being tested, glycine was left in Group 1 at 10 mg./liter because of the growth stimulation by the whole group of 12 amino acids. The effect of individual amino acids was not determined for Group 1, so glycine has regularly been part of Media 100 and 104.

The purpose of this study was to determine if the growth of firm white callus could be improved in both quantity and quality by the omission of glycine, hydroxyproline, or both from the supplements. All supplements were added at 10 mg./liter. At the end of the normal four-week passage, all callus cultures were again subcultured and grown for a second four-week passage on the test media. At the end of the second passage, the average wet weight of callus had increased from the initial 36 mg. to over 300 mg. on all test media. Table III shows that the differences were not significant according to Duncan's Test.

However, the fastest growing callus was on Medium 104H (No. 1) made with hydroxyproline but not with glycine (Table III). The slowest growing callus culture had both glycine and hydroxyproline (No. 4). On this basis, glycine was eliminated from the supplements but hydroxyproline was not. We felt that the 5% level of significance was too broad to catch the subtle callus changes visible on the different media. Other tests have confirmed these results, that the quality of firm white callus is enhanced by the addition of hydroxyproline but not glycine.

TABLE III

CALLUS GROWTH ON MEDIUM 104 (MG.), MADE WITH AND WITHOUT GLYCINE AND HYDROXYPROLINE, PLUS OR MINUS ONE STANDARD DEVIATION

Medium	Glycine	Hydroxyproline	Average Wet Weight[a]	GF[b]
1	-	+	367 ± 45[c]	10.1
3	+	-	358 ± 40	10.0
2	-	-	338 ± 79	9.5
4	+	+	302 ± 78	8.2

[a] Increment = total - initial wet weight per piece.

[b] Growth factor = (final - initial)/initial wet weight.

[c] Nonsignificant differences among all means at 5% DMT.

In a separate experiment, each of the stock cultures was grown on its own medium made without hydroxyproline in Media 100 and 104. The average wet weight increments and growth factors are given in Table IV, at four and five weeks after subculture. Ten of the fifteen original explants are shown in Fig. 4. In this experiment, Medium 100 (Fig. 4B) gave the fastest growth without hydroxyproline but with glycine, but also contained tryptophan.

TABLE IV

CALLUS GROWTH ON MEDIA 1, 100, AND 104 MADE WITHOUT HYDROXYPROLINE

Stock Medium	Four Weeks		Five Weeks	
	Av., mg.	GF	Av., mg.	GF
1	183	4.8	255	7.2
100	361	9.7	608	17.0
104	296	7.5	471	12.6

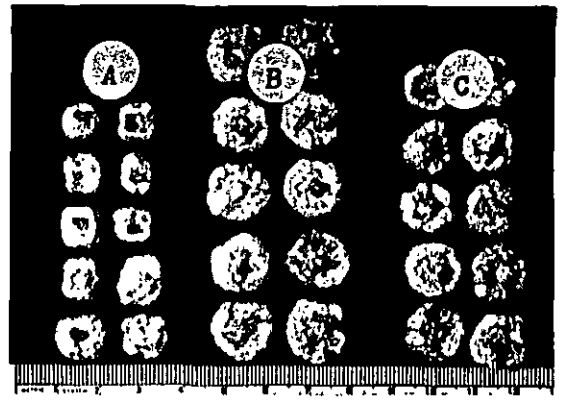

Figure 4. Stock Callus Grown for Five Weeks on Medium 1 (Fig. 4A), Medium 100 (Fig. 4B), and Medium 104 (Fig. 4C) Without Hydroxyproline. Media 100 and 104 had Glycine at 10 Milligrams per Liter

During the following passage, each stock callus was grown on the same media without hydroxyproline for four weeks. In the next passage, not only hydroxyproline was eliminated, but glycine as well. Callus 104 was also grown for four weeks on media made with either glycine or hydroxyproline and one medium had both. These averages and growth factors are given in Table V.

TABLE V

STOCK CALLUS GROWN ON MEDIA WITH AND WITHOUT GLYCINE AND HYDROXYPROLINE

Medium	Glycine	Hydroxyproline	Four Weeks Av. Wet Incr., mg.	GF
1	-	-	157	4.0
100	-	-	450	11.8
104	-	-	401	10.6
104	+	-	446	12.2
104	-	+	554	14.2
104	+	+	519	13.9

Again, callus growth slowed when both glycine and hydroxyproline were omitted and increased when they were added. However, the growth was faster when only hydroxyproline was added compared to the addition of glycine also.

On the basis of this series of experiments, we omitted glycine from our Media 100 and 104 but kept hydroxyproline in both. The average wet weight increments and growth factors from the two subsequent four-week passages are listed in Table VI, but show a drop in callus growth on all media. We believe this drop may be caused by seasonal variations, but we cannot say for sure because we did not continue running control cultures with both glycine and hydroxyproline.

TABLE VI

WET WEIGHT INCREMENTS (MG.) FOR TWO FOUR-WEEK PASSAGES ON MEDIA MADE WITHOUT GLYCINE BUT CONTAINING HYDROXYPROLINE

	First Passage		Second Passage	
Medium	Average Weight	GF	Average Weight	GF
1	248	6.3	65	0.9
100	312	7.9	337	8.8
104	361	9.1	267	6.8

There are probably other factors contributing to the general seasonal variations, but we may also find that glycine stimulates callus growth at a lower concentration. With the close of this project, we will curtail most growth studies, but a few refinements may be possible in our role of supplying callus tissue to students working on their thesis or Special Studies. We have supplied firm white callus to two students already, and we also use this callus for laboratory work in the course Plant Tissue Culture A-378. A limited amount of nutrient improvement is possible each month during subculture, with only a small additional

amount of time necessary. However, a student problem or a new project for aspen callus cultures would be necessary to study carefully the factors influencing seasonal growth of callus. We also feel that new callus should be initiated every 2-3 years, to insure a continual supply of callus without danger of chromosome changes during prolonged culturing.

In Fig. 5, one typical dish of stock callus is shown after 4.5 weeks on each medium in the dark. This was the condition of the callus last September, about nine months after the end of the series of experiments reported in Tables III to VI. The quality of firm white callus is good on all media (still made without glycine but with hydroxyproline in Media 100 and 104), and the growth on 100 and 104 is not significantly different. This is typical moderate growth, and the shoot initiation tests from this type of growth during the past 6-9 months have had very poor results. This is perhaps one-half of our normal fast growth recorded during the second and third years after callus initiation. This callus is now five-years old, and at the time of this photo (Fig. 5) the callus had been subcultured 55 times, including 42 times on Media 100 or 104 (1-23-67 to 9-17-71).

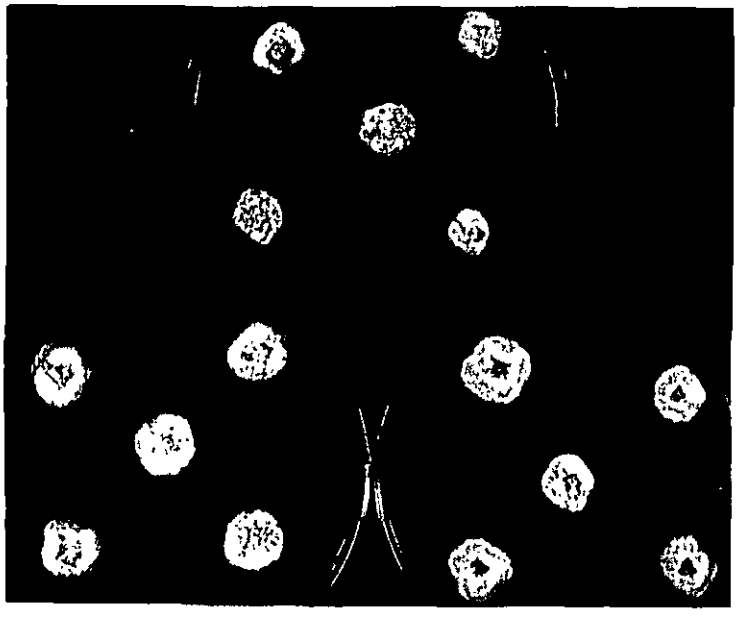

Figure 5. Callus of Triploid Quaking Aspen After 55 Subcultures to Medium 1 (Top), and Including 42 Subcultures on Medium 100 (Left), and Medium 104 (Right) from Callus Initiation on 1-23-67

STOCK CALLUS 104 GROWTH CURVE

For Special Studies A-291, Mr. Richard Smeltzer, a student at The Institute of Paper Chemistry, used firm white callus on Medium 104 to develop a quantitative isotope assay for auxin. The callus has never been given the natural auxin indoleacetic acid (IAA), but he found evidence that the callus makes its own IAA in measurable quantities about 5-6 weeks after subculture. This probably explains why older callus from subculture generally initiated fewer shoots than younger callus.

While we were providing callus each week for this study, we also made a growth study and shoot initiation test on which to terminate Project 2351. Callus was growing moderately fast, and explants were cut January 12, 1972 and placed five per dish on Medium 104 without glycine. All explants were weighed initially, and one set of 15 was oven-dried for our initial weight for subsequent ovendry calculations. Each succeeding week, 10 explants were weighed wet, the average piece was cut in two and used by Mr. Smeltzer for duplicate extractions, and the remaining 9 explants were oven-dried. The growth curve was plotted for nine weeks.

Figure 6 shows that, for moderate growth, callus on Medium 104 entered its exponential log phase of growth at about three weeks and continued for another four weeks. The rate of growth slowed slightly at six weeks after subculture, but after the high at seven weeks, dropped quickly during the eighth and ninth weeks. During the same growth study, the oven-dried weights slowly increased to the seven-week high then dropped, but only slightly. After seven weeks, the decrease in wet weight probably was caused by dehydration entirely, with very little loss of dry weight. The standard deviations of the dry weights were too

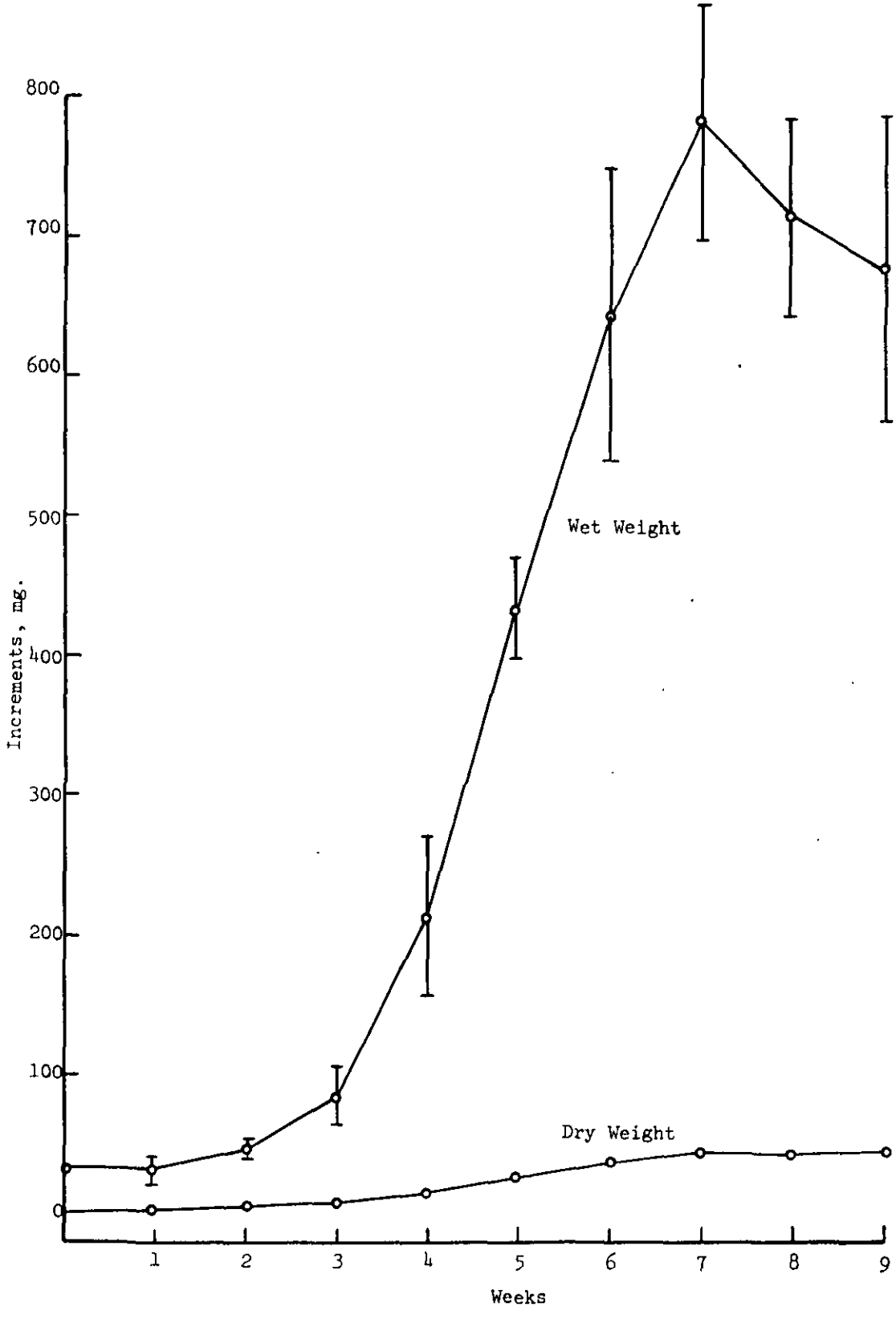

Figure 6. Growth Curve of Aspen Callus on Medium 104 (Without Glycine) in the Dark at 27°C., Measured Weekly from 1-12-72 to 3-15-72 for Nine Weeks

small to show in Fig. 6, but are given in Table VII with the percentages of dry weight for each week.

TABLE VII

INCREMENT WET AND DRY WEIGHTS (MG.) OF ASPEN CALLUS MEASURED WEEKLY ON MEDIUM 104 IN THE DARK

Week	Ending Date	Increments[a] Wet Weight	Dry Weight	Percent WW/DW
0	1-12-72	32 ± 2	1.3 ± 0.2	4.1
1	1-19	31 ± 9	2.5 ± 0.4	8.0
2	1-26	48 ± 6	5.4 ± 0.6	11.2
3	2-2	83 ± 22	8.5 ± 1.3	10.2
4	2-9	213 ± 59	14.9 ± 2.4	7.0
5	2-16	432 ± 36	25.4 ± 2.1	5.9
6	2-23	643 ± 102	35.7 ± 5.0	5.6
7	3-1	784 ± 87	42.9 ± 3.7	5.5
8	3-8	713 ± 71	40.2 ± 2.8	5.6
9	3-15	677 ± 110	44.3 ± 7.0	6.5

[a] Fifteen inocula were measured for the initial wet and dry weights, then ten pieces were weighed each week for wet increments and nine pieces for dry increments. The average wet and dry increments are reported in milligrams plus or minus one standard deviation.

$$\text{Average} = \frac{\text{Sum of increment weights}}{\text{Number of samples}}$$

$$\text{Standard deviation} = \sqrt{\frac{\Sigma x^2 - \frac{(\Sigma x)^2}{N}}{N-1}}$$

SHOOT INITIATION FROM OLD CALLUS T-2-56

During the past year, the initiation of shoots from aspen callus continued to be of primary interest. Our stock cultures are now five-years old, first producing complete plants when one-year old, trees when two-years old, and numerous smaller shoots when three-years old. However, during the past two years, the general callus growth and ability to produce shoots appears to have declined. This is in contrast with growth during the second year of fast growth on Medium 104, moderate callus growth on Medium 100, and slow growth on Medium 1. In the second and third year, seasonal fluctuations ranged from slow to fast in an unpredictable manner, although growth curves were fairly consistent for the same seasons, for the two years monitored for each culture (Report Ten, page 11).

Last year we appreciated the fact that callus growth was declining; and with growth, the frequency of shoot initiation was also decreasing. During the past three years callus on Medium 1 has put out few, if any, shoots, although the first complete plants were from this callus. Subtle biochemical or genetic changes are probably responsible for the callus decline, but rather than trying to increase growth and shoot initiation with nutrient studies on the old callus, we feel that new callus should be initiated every two years. A few plates of old callus should also be kept as controls and for special studies, but a fresh supply of firm white, fast-growing aspen callus should be available at all times.

In addition to new callus on the old media, we started callus on the same basic Medium 1 with different auxins, because of the several reports among other workers who found that the auxin 2,4-D (2,4-dichlorophenoxyacetic acid) apparently inhibits the formation of shoots from callus. Sugarcane callus was initiated on medium containing 2,4-D, as well as on media with other auxins like

IAA and NAA (naphthaleneacetic acid) (20). Callus grew faster with 2,4-D, but roots and shoots grew better on callus initiated with IAA.

In order to study the correlation between auxin and subsequent shoot initiation, we devised two main types of experiments. In the first, we initiated new callus on Medium 1 with our regular level of 2,4-D (0.04 mg./liter), and also on the same medium using 10 mg./liter IAA. In the second type of experiment, old callus from aspen stock cultures was transferred to Media 1, 100, or 104, and some callus was tested monthly for shoot initiation. In these two studies, we compared shoot initiation from the same triploid clone (T-2-56) of quaking aspen, from callus growing on different media. In the other approach, we initiated callus from a second clone (T-38-59) of triploid quaking aspen, on media containing 2,4-D or IAA. These studies will be reported separately in the following sections, and will represent the most typical of each type of experiment.

To review briefly, callus growth and shoot initiation are two separate processes carried out on two different media. However, the number of shoots and their vigor appears to be affected in many ways by the type of callus growth preceding shoot initiation. Generally, callus is grown on a balanced medium in the dark, on a medium containing two growth hormones, on auxin and a cytokinin. In aspen Media 1, 100, and 104 the auxin is 2,4-D and the cytokinin is kinetin. In order to initiate shoots, the auxin is omitted and kinetin is replaced by 0.05 or 0.10 N^6-benzylaminopurine (BAP), giving Medium BAP-.05 or BAP-.10.

The shoot initiation medium is usually modified Medium 1, and does not contain the supplements normally in Media 100 or 104. However, past work has shown that callus growth on Media 100 or 104 (with or without tryptophan) increased both the quality and quantity of shoots initiated. Shoots are initiated

in the dark, and after one week or so they are placed in the light on the original auxin medium, where they turn green within a few days and sometimes grow roots after two weeks. Our purpose in substituting IAA for 2,4-D for callus growth was to force shoot induction within a few months, instead of one year or more after callus initiation.

Another difference between callus growth and shoot initiation is that on BAP media (without auxin), firm white callus does not grow much, if any. Shoots apparently are differentiated in nests of cells which divide but do not appreciably enlarge. We have had enough experience not to expect shoots if the inocula placed on BAP medium renews growth. However, we expect shoots if the callus remains white but does not grow. Shoots generally are visible at the sides or undersides of callus pieces 3-4 weeks after they are placed on BAP medium. Shoots initiated after 1-2 weeks are usually the most vigorous, but those growing after 5-6 weeks are generally stunted and quickly die. For fast growing callus, shoots are more vigorous on BAP-.05, but the less vigorous shoots initiated with BAP-.15 will root more easily.. All surviving trees came from shoots initiated within 3-4 weeks on BAP-.05 (16). One of our students, Mr. Richard Smeltzer, is very interested in correlating the chemical changes within the growth centers of differentiation with the frequency and vigor of shoot initiation. By our arbitrary definition, a vigorous shoot is one exceeding 5 mm. one week after it is first observed. Small shoots are 3-4 mm. long, and any stunted shoots less than 3 mm. do not survive. One callus piece may have one or two vigorous shoots, but we have counted up to a couple of dozen stunted shoots per piece. But again, it has only been the most vigorous shoots that have rooted on the callus to form complete plants. We also have two trees from rooted excised shoots.

DIFFERENT LEVELS OF 2,4-D AND KINETIN

The auxin effects of 2,4-D are 10-20 times those of natural auxin IAA, probably because there are no natural destructive systems in plant callus for 2,4-D. We can maintain firm white callus with amounts as low as 0.004-0.04 mg./liter (parts per million) of 2,4-D, and can give callus 10-50 mg./liter IAA without causing significant growth inhibition.

In the first experiment reported here, we tested the range of 2,4-D that can be used in Medium 104 to grow firm white aspen callus. We suspect that without a destructive system, 2,4-D might be building up in the callus during successive subcultures, eventually arriving at a concentration inhibitory not only to subsequent shoot initiation but also to callus growth. We transferred callus from Medium 104 (with 0.04 2,4-D and 1 kinetin) to a series of Medium 104 made with 0, 0.004, 0.04, and 0.4 mg./liter 2,4-D at 1 kinetin, and also one medium with 0.04 2,4-D and 2 kinetin. Fifteen inocula were grown on three dishes in the dark for three monthly passages. Wet weights were not recorded, but visual comparisons can be seen in Fig. 7, showing the best five pieces of callus from each treatment. This callus was subcultured to BAP-.1 medium when five-weeks old. The ranking of callus growth and the subsequent number of callus pieces producing one or more shoots are found in Table VIII.

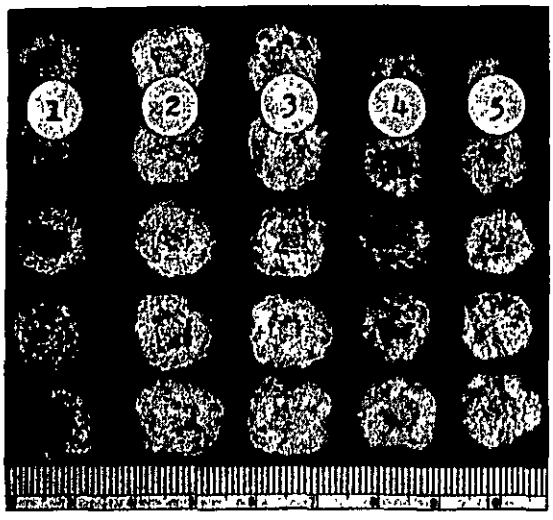

Figure 7. Callus of Triploid Quaking Aspen T-2-56 After the Third Monthly Passage on Medium 104 Made with the Levels of 2,4-D and Kinetin Shown Below:

	2,4-D	Kinetin
1	0	1
2	0.004	1
3	0.04	1
4	0.04	2
5	0.4	1

TABLE VIII

MEDIUM 104 WITH DIFFERENT LEVELS OF 2,4-D AND KINETIN (MG.)

Medium	2,4-D	Kinetin	Quality Ranking	Pieces with Shoots[a]	
1	0	1	Second	0	0%
2	0.004	1	First, equal to 3	2	13%
3	0.04	1	First, equal to 2	11	73%
4	0.04	2	Third	10	67%
5	0.4	1	Fourth	3	20%

[a]Percentages are based on 15 inocula per treatment.

On the basis of these results, the regular levels of auxin and cytokinin used in Medium 104 appear to be optimum for the concentrations tested, not only for callus growth but also for subsequent shoot initiation. Callus was able to survive for three monthly passages without added 2,4-D, and the amount of quality callus did not decrease as much as might be expected. One explanation is that residual 2,4-D continued to influence growth to the third generation. But a more

likely answer is that the callus produces its own natural IAA. A combination of the two is also possible. Callus grew about equally well with either 0.004 or 0.04 mg. 2,4-D, but subsequent shoot initiation from callus grown with 0.04 was five times that grown with 0.004 2,4-D.

Another interesting contrast is that callus grown with 0.04 2,4-D, with either 1 or 2 mg./liter kinetin, produced shoots on about the same number of callus pieces, but the growth and quality of callus grown with 2 mg. kinetin was much lower than with 1 mg. kinetin. The highest level of 2,4-D, at 0.4 mg./liter, inhibited both growth and shoot initiation. Contrary to our hypothesis, 0.04 2,4-D and 1 kinetin apparently are the optimum levels for Medium 104 for both callus growth and subsequent shoots production.

AGE OF CALLUS FROM SUBCULTURE

Several times in the past we have grown stock callus for different lengths of time after subculture, in order to determine the correlation of this factor with subsequent shoot initiation. However, we continued to maintain stock cultures at four-week passages as controls. In this test, we grew separate lines of the three stock cultures on Media 1, 100, and 104 for 4, 5, or 6 weeks for several passages. The number of new shoots on Medium BAP-1 was recorded each week for eight weeks until 11-9-70. In Table IX are shown the number of shoots per piece at the end of eight weeks. Medium BAP-1 was made with 1 mg./liter instead of 0.1 mg. BAP.

From this study, it is obvious that the five-week old callus on Medium 100 gave the best shoot initiation, both in the total number of inocula with one or more shoots, and in the total number of shoots produced. During the past few years, Callus 100 occasionally grew faster and produced more shoots than Callus 104.

However, the reverse was generally true. Callus on Medium 1 has not produced shoots for the past three years.

TABLE IX

NUMBER OF SHOOTS PER PIECE AFTER EIGHT WEEKS ON MEDIUM BAP-1

Age of Stock, weeks	Stock Callus	Inoculum Number on BAP-1															Total Shoots	Pieces With Shoots
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		
Four	1	None															0	0
	100	None															0	0
	104	2 shoots on one piece															2	1
Five	1	None															0	0
	100	0	1	1	0	1	1	2	9	2	0	2	2	2	0	3	26	11
	104	0	2	1	2	0	0	0	0	2	1	1	0	2	1	3	15	9
Six	1	None															0	0
	100	1	0	0	0	0	3	0	0	0	0	5[a]	0	0	0	0	9	3
	104	2	1	3	0	0	1	3	2	0	0	0	0	0	0	0	12	6

[a]Four vigorous shoots transferred to Medium 104 in the light. However, the medium eventually became contaminated and was discarded.

MEDIA 1, 100, AND 104 WITHOUT GLYCINE

In March, 1971, in the same month that callus with different levels of 2,4-D (Fig. 7) was placed on BAP medium, stock callus from Media 1, 100, and 104 (without glycine) was also subcultured to Media BAP-.1 and BAP-.5. This callus had gone through 49 passages from initiation, including 36 on Media 100 and 104. The number of pieces with one or more shoots is given in Table X for each treatment five weeks after subculture to BAP media.

In April, 15 inocula were again transferred from Media 100 and 104 to Medium BAP-.1. After five weeks in the dark, four pieces (27%) had shoots from Medium 100 and three pieces (20%) had shoots from Medium 104. However, all shoots were less than 2-3 mm. tall and were discarded.

TABLE X

NUMBER OF INOCULA WITH SHOOTS FROM STOCK CALLUS AFTER FIVE WEEKS

Medium	BAP	No. of Inocula	Pieces with Shoots		
			Number	Percent	
1	.1	10	0		
	.5	10	0		Rapid growth
100	.1	25	4	16%	1-2 mm. shoots
	.5	25	4	16%	2-4 mm. shoots
104	.1	25	0		
	.5	25	0		

During the past year, stock callus has been transferred to BAP-.05 and BAP-.1 media every few months. However, no shoots have grown from any of the three cultures on Media 1, 100, or 104, where the last two receive hydroxyproline but not glycine. Even during the last growth study reported in Fig. 6, and completed this past March 15, 1972, no shoots were initiated from Callus 104. At this time, though, seasonal factors other than the omission of glycine probably can explain shoot inhibition.

GLYCINE-HYDROXYPROLINE AND AGE EFFECTS OF INOCULA FROM SUBCULTURE

Glycine was omitted from the supplements in Media 100 and 104 in November, 1970. Stock callus subcultured in September had both glycine and hydroxyproline in these two media. In October, weekly subcultures were started for callus on the three media, and inocula were placed on media with different levels of BAP. In addition, some callus from Medium 104 had been grown for one passage on Medium 104 without hydroxyproline but with glycine. Callus from Medium 104H was placed on Medium BAP-.1 when four-weeks old, as well as on Medium 104 BAP-.1, which contained the supplements but no auxin or kinetin. The total number of pieces with shoots is given in Table XI.

TABLE XI

CALLUS PIECES FROM STOCK CULTURES WITH ONE OR MORE SHOOTS AFTER FIVE WEEKS

Age at Subculture, weeks	Stock Medium	Number of Inocula	BAP Media, mg./liter	Number Pieces with Shoots	Remarks
4	1	10	0.1	0	
	100	10	0.1	0	
		10	0.1-10⁴	0	
	10⁴	5	0.1	0	
		5	0.1-10⁴	0	
	10⁴H	5	0.1	0	5 with roots
		5	0.1-10⁴	0	4 with roots
5	1	15	0.1	0	6 with roots
	100	15	0.1	1	
		10	0.5	0	Good growth
		10	1	0	Good growth
		10	3	0	
		10	5	0	
	10⁴	15	0.1	5	1 vigorous
		10	0.5	5 on 5	Callus feathery
		10	1	0	Good growth
		10	3	0	
		10	5	0	
6	100	10	0.1	6	
		10	0.5	1	
		10	1	0	
		10	3	0	
		10	5	0	
	10⁴	10	0.1	2	
		10	0.5	8	
		10	1	1	
		10	3	0	
		10	5	0	
7	100	10	0.1	1	
		10	0.5	0	
		10	1	0	
		10	3	0	
		10	5	0	
	10⁴	10	0.1	2	
		10	0.5	4	
		10	1	0	
		10	3	0	
		10	5	0	

TABLE XI (Continued)

CALLUS PIECES FROM STOCK CULTURES WITH ONE OR MORE SHOOTS AFTER FIVE WEEKS

Age at Subculture, weeks	Stock Medium	Number of Inocula	BAP Media, mg./liter	Number Pieces with Shoots	Remarks
8	1	10	0.1	0	
	100	10	0.1	0	
		5	0.5	2	
		5	1	0	
		5	3	0	
	104	10	0.1	3	
		5	0.5	3	
		5	1	0	
		5	3	0	
		5	5	0	
9	104	10	0.1	2	
10	104	10	0.1	0	

At five weeks after subculture, the inocula cut from Callus 104 was firm white only on BAP-.5 and BAP-1, but the callus pieces were significantly larger on BAP-.5 than on all other BAP media, and shoots were very small and numerous.

In only one test of four-week old callus was 104H compared with Callus 104, 100, and 1. However, no shoots were initiated from four-week old callus from any stock cultures, either on regular BAP medium or BAP supplemented with Medium 104 amino acids and nucleotides. No shoots were initiated from Medium 1 callus on any BAP medium. In weeks 5 to 8, where Callus 100 was compared with Callus 104, shoots were observed as follows in Table XII (condensed from Table XI).

TABLE XII

SHOOTS PRODUCED FROM CALLUS 100 AND 104

Week	Stock	Inocula	BAP	Pieces with Shoots	Percent
5	100	15	0.1	1	7
	104	15	0.1	5	33
		10	0.5	5 on 5	100
6	100	10	0.1	6	60
		10	0.5	1	10
	104	10	0.1	2	20
		10	0.5	8	80
		10	1.0	1	10
7	100	10	0.1	1	10
	104	10	0.1	2	20
		10	0.5	4	40
8	100	10	0.5	2	20
	104	10	0.1	3	30
		5	0.5	3	60

Summation	BAP-.1	BAP-.5	BAP-1	Total	Percent
100	8	3	0	11	25
104	12	20	1	33	75
	20	23	1	44	

04,

The data in Tables XI and XII are probably insufficient for analysis, but they tend to support a rather persistent hypothesis in this laboratory, that natural IAA is produced in callus as it ages, thus requiring higher levels of BAP for shoot initiation with increased age of the callus from subculture. Support for this hypothesis was also found during the A-291 student research, by Mr. Richard Smeltzer, during the fall and winter quarters of 1971-72, who found IAA in six-week old callus given 2,4-D but never IAA.

In order to show the number of shoots per piece and their distribution according to size of callus piece, the five pieces in one dish from each treatment were weighed for total wet weight, and the number of shoots per piece were counted. These data are presented in Table XIII.

TABLE XIII

TOTAL WET WEIGHT (MG.) AND SHOOTS PER PIECE,
EIGHT WEEKS AFTER SUBCULTURE

Stock	BAP-.1		BAP-.5		BAP-1		BAP-3		BAP-5	
Callus	Weight	Shoots	Weight	Shoots	Weight	Shoots	Weight	Shoots	Weight	Shoots
104	48		284	3	370		149		124	
	92		412	5	310		227		170	
	69		318	1	342		195		50	
	100		338	6	347		222		163	
	92	1	329	5	394	1	208		167	
Total	400	1	1680	20	1763	1	1001	0	653	0
Average	80	1	336	4	353	1	200	0	131	0
100	125	2	216		357		168		119	
	300		390		545		238		199	
	208	2	300		309		315		250	
	262		258		482		194		206	
	142	3	305		536		289		71	
Total	1036	7	1494	0	2229	0	1204	0	844	0
Average	207	1.4	299	0	446	0	241	0	169	0
TOTAL	1436	8	3174	4	3992	1	2205	0	1497	0
AVERAGE	144	0.8	317	0.4	399	0.1	220	0	150	0

SHOOT INITIATION FROM NEW CALLUS

In the previous section, we reported recent shoot-initiation experiments with stock callus which was four or five-years old from callus initiation. For possible reasons already discussed, the callus appears to be declining both in growth rate and in the ability to produce shoots. Thus, in addition to testing shoot initiation from old callus, new callus was also initiated from triploid quaking aspen T-2-56, as well as from triploid quaking aspen T-38-59. New types of media were also used to start callus, made with IAA instead of 2,4-D, although regular 2,4-D media were used as controls. We also tried variations of a different basic medium based on Murashige and Skoog (20), rather than on our usual Wolter and Skoog (9) medium.

Callus has been initiated from different natural clones of triploid quaking aspen several times during the past 3-4 years. In Report Ten, the superior callus growth from Clone T-38-59 was reported on page 19. It was initiated equally well on Media 1 and 100, but not well on Medium 104. The best firm white growth was on callus initiated on Medium 1 then transferred to Media 100 or 104. Slow-growing yellow callus was initiated on Media IAA-1 and IAA-10, made by substituting either 1 or 10 mg./liter IAA in Medium 1 for 2,4-D. Callus was also initiated on the same media from T-2-56.

The several goals of this series of experiments included the comparison in callus growth and shoot initiation between T-2-56 and T-38-59 on similar media, and also to test the early shoot production from callus of both clones initiated with IAA or 2,4-D. Our hypothesis was that callus initiated with IAA should produce shoots earlier than callus initiated with 2,4-D.

TRIPLOID QUAKING ASPEN CLONES

On March 5, 1970, new callus was initiated from Clones T-2-56 and T-38-59, from rooted root-sprouts grown in the greenhouse to about one foot in height. Segments 2-3 inches long were sterilized in bleach plus Tween-20 for 15 minutes. After three rinses with sterile water, 5-7 mm. internodal segments were cut and placed base up in several media, including Media 1, C.04 (Medium 1 plus 15% coconut milk), and Medium 1 with 10 mg./liter guanylic acid (nucleotide). At the end of one month, callus was subcultured to fresh media. Some callus initiated on Medium 1 was left there, but some was also transferred to Medium 104. Callus on Medium 1G (with guanylic acid) was subcultured to Medium 1, and callus on Medium C.04 was subcultured to Medium C.04.

Callus was also initiated from the same clones on April 16, on Medium 1 and three variations. The four media contained combinations of 0.04 mg. IAA or 2,4-D, with or without supplements of 100 mg. asparagine and 0.025 mg. each of copper sulfate, cobalt chloride, and sodium molybdate. Stock callus from the thirty-ninth passage on Medium 1 was also subcultured to these MW-1 media. Callus on Medium MW-1-1 (made with 0.04 IAA) was transferred to Medium 1 with 1 mg. IAA, then to 10 mg. IAA over the next two passages. Callus on Medium IAA-10 was maintained in the dark as firm yellowish-white tissue, and monthly tests were made on BAP medium for shoot initiation.

On May 4, 1970, callus was initiated from both triploid clones on the MW-1 media. Callus was also initiated on stock media 1, 100, and 104, as well as on new media based on Murashige and Skoog. Media MS-1 and MS-2 both had 4 mg. IAA, and either 0.64 or 2.56 mg. kinetin, respectively.

The best firm white callus from both clones was on Medium 1 made with 2,4-D. Callus on IAA medium was maintained on Medium IAA-10 and some was transferred to media containing 10, 25, or 50 mg./liter IAA. However, the best growth was on IAA-10.

Beginning with the first passage after initiation, most callus cultures from Clones T-2-56 and T-38-59 were tested on Medium BAP-.1 for shoot production. At the same time, stock callus of T-2-56 was also tested to compare shoot initiation from callus of different age from initiation. Usually, only five inocula per one dish of BAP medium was used, because of the small amount of surviving newly-initiated callus.

From the newly-initiated callus, the first shoots appeared on Callus T-2-56 during the seventh passage from initiation on 3-5-70. The callus had been initiated on Medium 1, transferred to Medium 104 for six passages, then subcultured to Medium BAP-.1 on 10-13-70. Three weeks later, two pieces had vigorous shoots in the dark, and after another week four out of five inocula had shoots. The inocula did not put out new callus growth, which is normal when shoots are produced. But there were many aerial, nonfunctional roots from the callus which are normally not produced at the same time that shoots are initiated. A "balanced" ratio of auxin/cytokinin produces rapid growth of firm white callus, and an increase in auxin promotes root growth while an increase in cytokinin produces shoots. However, functional roots from callus and shoots usually do not grow until two weeks after the callus is returned to Medium 104 in the light.

In Fig. 8B, the callus from T-38-59, on Medium BAP-.1, grew 2-3 times as fast as on Medium 104 (Fig. 8D), which is rather difficult to explain without further data. Figure 8C shows newly-initiated T-2-56 callus on Medium 104 during the ninth passage. Newly-formed T-38-59 callus continued growing as firm white callus when transferred from Medium 104 to Medium BAP-.1, and the following month the same results were obtained. Shoots were initiated only from T-2-56 callus, but firm white callus grew from T-38-59. This could indicate that the T-38-59 callus was making natural IAA that was inhibiting shoot growth and promoting callus growth. This relationship is illustrated in Fig. 8, of three-week old callus from the 12-22-70 subculture of T-2-56 (Fig. 8C) and T-38-59 (Fig. 8D) during the ninth passage on Medium 104. In Fig. 8A and 8B, respectively, are shown shoots and non-functional aerial roots on T-2-56 callus, but T-38-59 callus grew rapidly and did not grow shoots. No shoots were produced by T-38-59 callus at any time from the March or April initiations.

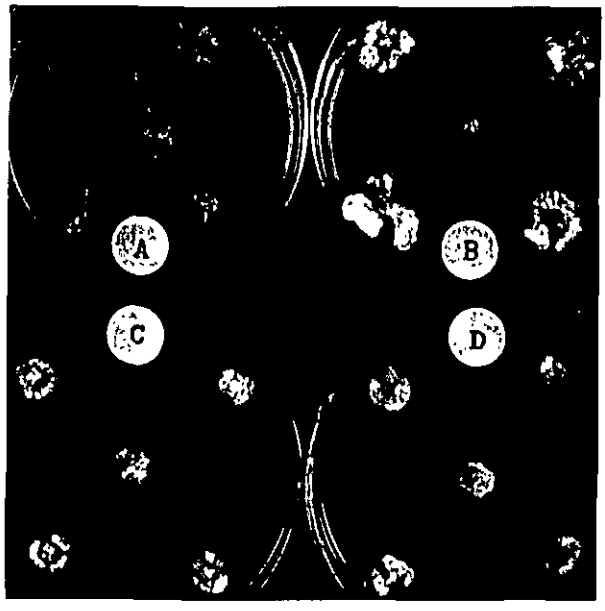

Figure 8. Newly-Initiated Callus of T-2-56 (Fig. 8C) and T-38-59 (Fig. 8D) After Three Weeks on Medium 104, During the Ninth Monthly Passage from Initiation. Inocula from the Seventh Passage is Shown After Six Weeks on Shoot-Initiation Medium BAP-.1 from T-2-56 (Fig. 8A) and T-38-59 (Fig. 8B). Shoots were Initiated on T-2-56 Callus but not from T-38-59 Callus

However, one small shoot from T-38-59 was recovered in January, 1971, seven months after callus was initiated on 5-4-70. The callus had been initiated on Medium 1, then maintained for five passages on Medium 104 (Table XIII). Also, from older Callus T-38-59 initiated 4-15-69, another shoot was produced on 11-16-70, from callus 15-months old from initiation. This callus was also initiated on Medium 1 and maintained for sixteen passages on Medium 104.

Unfortunately, all shoots produced from newly-initiated Callus T-2 or T-38 were weak and did not survive. They were placed in the light on Medium 104 or BAP-.1, and turned green and some callus grew roots. But no shoots rooted and no complete plants were formed. Shoot initiation was also attempted on Medium 104-BAP-.1, containing the supplements but no auxin. Neither were shoots obtained on Medium BAP-.05.

On the basis of these data, seven months after callus initiation seems to be the earliest that shoots can be produced from callus of either triploid quaking aspen T-2-56 or T-38-59, under the conditions described. However, the frequency was too low to calculate and no complete plants or trees were recovered. Our media were designed for Callus T-2-56, so it is not surprising that the T-38-59 callus behaved differently. When auxin was withheld from the rapid-growing T-38-59 callus, growth was accelerated and no shoots were produced. On the other hand, when auxin was withheld from T-2-56, it did not grow new callus but occasionally grew shoots. Evidently, the T-38-59 callus makes its own IAA auxin.

In Table XIV, the average total wet-weights of five inocula per treatment are given, along with the average number of shoots per piece of Callus T-2-56 and T-38-59, initiated in early 1970 and subcultured for 5-9 passages on different media. Comparisons are shown for stock cultures on Media 1, 100, or 104, as well

as for stock callus transferred to Medium BAP-.1 for shoot initiation. Figure 9 shows the relatively slow growth of Callus T-38-59, on four media after five weeks of the fifth passage, from callus initiation on 4-16-70.

TABLE XIV

FRESH WEIGHT AND SHOOT PRODUCTION FROM NEWLY-INITIATED AND OLD CALLUS

Date of Initiation	Callus	Stock Medium	No. of Passages	Test Medium	Average per Piece Weight, mg.	Shoots
			Newly-Initiated Callus		Four Weeks	
3-5-70	T-2	104	9	104	434	0
			7	BAP-.1	205	2.4
	T-38	104	9	104	442	0
			7	BAP-.1	549	0
4-16-70	T-38	1	8	1	61	0
			6	BAP-.1	419	0
		IAA-10	7	IAA-10	284	0
			5	BAP-.1	746	0
5-4-70	T-38	1	7	1	60	0
			5	BAP-.1	159	0
		100	7	100	273	0
			5	BAP-.1	471	0
		104	7	104	372	0
			5	BAP-.1	171	0.2
			Old Callus		Six Weeks	
1-23-67	T-2	1	44	1	324	0
				BAP-.1	--	0
		100	44	100	569	0
				BAP-.1	--	0.6
		104	44	104	587	0
				BAP-.1	--	0.1

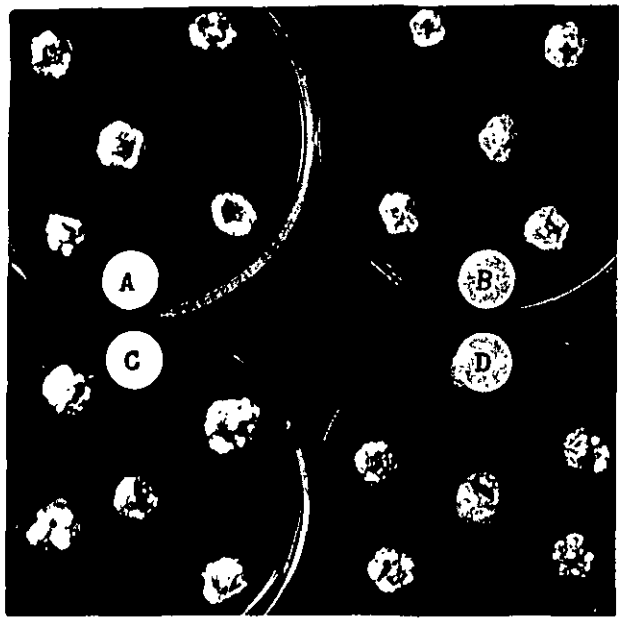

Figure 9. T-38-59 Callus on Medium IAA-10 (Fig. 9A), Medium 1 (Fig. 9B), Medium 100 (Fig. 9C), and Medium 104 (Fig. 9D) After Five Weeks in the Dark, During the Fifth Passage from Initiation on Medium 1

OTHER ASPEN CALLUS CULTURES

In Table V, Report Ten, page 22, 14 species and hybrids are listed which were used to initiate callus cultures TH (tall hybrids). Callus was started on Medium 1 then maintained on Medium 104. In addition, cultures of a dozen or so other aspen species, hybrids, or putative homozygous diploids (from a haploid study in another project) were also all maintained on Medium 104 in the dark at 27°C. Several were supposedly triploids or tetraploids, respectively, formed from crosses using pollen from a tetraploid male (Ta-10) European aspen, or using unreduced triploid (3n) pollen from a triploid aspen to pollinate normal quaking aspen female flowers.

Callus Ta-10, from the tetraploid European aspen, produced shoots during its twenty-third passage after initiation in December, 1967 (11). Roots were also initiated from the callus, and gave rise to many shoots that grew their own roots and were separated from the callus as complete plants. These,

as well as later rooted-shoots have been planted in soil in the greenhouse. Now there are a dozen or more trees, up to one foot in height, that will later be planted out to compare their growth with trees of the same species that have been vegetatively propagated from branch cuttings.

One other culture also produced one complete plant. Under another project on tree improvement, pollen was heated to 50°C. for 4 minutes after it was collected from a white poplar tree (P. alba). The weakened pollen was used to pollinate diploid quaking aspen in the Cross XT-A-29-68 in 1968. Most of the five pubescent plants from 28-mesh seed (large seed) were prostrate in form. Tree 4-68 had chromosome counts ranging around the diploid number of 38, and also had hairy characteristics of the male parent indicating its hybrid origin. Callus was initiated from Tree 4-68 on 11-18-69 on Medium 1, then maintained on Medium 104. On 12-8-70, callus was subcultured to Medium BAP-.1, during its thirteenth passage, and one shoot was formed on 1-14-71 that was transferred to Medium 104 containing 0.03% IBA (indolebutyric acid), and was placed in the light on 3-16-71. The shoot elongated by the end of April and had grown a 6-cm. long root by May, when it was planted in soil and placed in the growth chamber 5-18-71. This tree is now growing well in the greenhouse and is nearly one-foot tall.

Shoots were produced on other callus that did not root or become complete plants, but so far only three aspen species have been reproduced from callus to trees. The fourth callus tree (Tree XI), from the first group of four, had callus initiated from one small side branch. After 22 passages, this callus grew shoots on five of ten inocula after six weeks on Medium BAP-.05, in November, 1971. The callus with the shoots was transferred to Medium 104 in the light, but none of the shoots rooted and all were discarded.

One of the tall hybrid cultures (TH-4) was initiated from the tallest plant in the nursery from Cross XCa-G-1-69 (Ca-2 x G-1-58), on Medium C.04 on 11-12-69. After 23 passages on Medium 104, one inoculum out of 10 had many small shoots, but none rooted. TH-14 (Ca-2) callus also put out shoots under light on Medium 100, but none rooted. The callus from gray poplar (P. canescens) is not sensitive to light, as is callus from quaking aspen, and callus turns green easily in the light.

In November, 1971, all firm white callus on all aspen cultures were put out on Medium BAP-.05 for one last shoot-initiation test. It was at the end of this test that the Tree XI callus and the TH-4 callus described above put out shoots. During the same test, Callus Ta-10 grew shoots from 9 of 10 inocula during its forty-sixth passage. One culture was Cold LC, from firm yellow T-2-56 callus grown in liquid culture, given a cold treatment, then grown as soft yellow callus for 69 passages. This was our oldest culture, but only one or two shoots have ever been produced. At the completion of this test in 1971, all callus cultures were discarded, except stocks of T-2-56 on Media 1, 100, and 104, the best callus from Ta-10 in its fiftieth passage, and some cultures in the light that began as cell suspensions. These cultures will be maintained and built up when needed for academic studies.

GREEN CALLUS CULTURES OF T-2-56

In closing this Final Report, it is a pleasure to announce the first growth of green callus cultures of triploid quaking aspen T-2-56. On the dozens of modifications of the Wolter and Skoog medium, T-2-56 callus has always been sensitive to light and grew as firm white callus only in the dark. In connection with another project involving conifer callus, we used liquid medium based on the Murashige and Skoog medium (18), containing 5 mg./liter β-naphthoxyacetic acid and 0.1 mg./liter N^6-benzylaminopurine as the growth hormones (21). Firm white T-2-56 callus was subcultured from the forty-fourth passage on Medium 104 into liquid medium MSNBA-10, and placed in a 25 x 125 cm. culture tube on a Rollardrum at 20 r.p.m., under tungsten-fluorescent light at about 200 ft.-c. The tube was wrapped in foil for one month, allowing no light to reach the callus. During the second month, the foil was removed and the callus turned dark green in 16 hours of light alternating with 8 hours of darkness.

Some green callus was transferred to agar plates of Medium MSNBA-10 and Medium 100 in the light. On Medium 100, green nodules 5-mm. in diameter turned red, so this callus was transferred to Medium MSNBA-10, where it reverted to a light-green coloration. Some of this callus is now on Medium BAP-.1 in the dark for shoot initiation, and other callus will be placed on MSNBA medium without auxin. However, no shoots have yet developed from the green callus. Our hope is that the growth rate of green callus can be accelerated with high auxin to produce very soft callus that can be used for cell suspensions. The firm white callus will not dissociate in liquid and forms very poor cell suspensions. But the green callus may be the means of obtaining cell suspensions that may eventually permit the reproduction of trees from single cells.

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1. Mathes, M. C. Antimicrobial substances from aspen tissue grown in vitro. Science 140:1101-2(1963).

2. Mathes, M. C. The culture of isolated triploid aspen tissue. Forest Sci. 10:35-8(1964).

3. Mathes, M. C. The use of isolated plant tissue in studies related to forest genetics. Tappi 47:710-13(1964).

4. Mathes, M. C. The in vitro formation of plantlets from isolated aspen tissue. Phyton 21:137-41(1964).

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19. Winton, L. L. Preparations of stained microscope sections. The Institute of Paper Chemistry *Genetics and Physiology Notes* no. 10, 1970.

20. Winton, L. L. Tissue culture propagation of European aspen. Forest Sci. 17:348-50(1971).

21. Winton, L. L., and Mathes, M. C. Aspen callus. In Methods and applications of tissue culture. Section II. Primary Cultures. (Invited paper to be submitted to Academic Press.)

22. Einspahr, D. W., and Winton, L. L. Genetics of quaking aspen. In Genetics of important North American forest tree species. (Invited chapter submitted for publication.)

Related Papers From Other Projects

23. Winton, L. L. Callus and cell cultures of Douglas-fir. Forest Sci. 18: (June, 1972 – in press).

PRESENTATIONS ON THE CALLUS TREE

1969	January 15	Project 1800 Meeting, IPC Annual Report to Cooperators.
	April 8	Press Conference on Callus Tree: Grummer, Van Horn, Einspahr, and Winton.
	May 8	Executives' Conference, IPC. Display.
	August 5	Eighth Annual Conservation Seminar for Teachers, Eagle River — Trees for Tomorrow Camp. Slide talk.
	August 18	World Forestry Congress (Washington, D.C.) North Central U.S. Tour. Holiday Inn, Appleton. Slide talk.
	September 31	XI International Botanical Congress, Seattle, Washington. "Aspen trees from callus tissue," Paper no. 1174.
	October 20	Science Seminar, IPC. Special discussion with plant physiology students from Lawrence University. Slides.
1970	January 6	Science Seminar, Sheboygan North High School. Slide talk.
	January 21	Project 1800 Meeting, IPC Annual Report to Cooperators.
	May 12	Fifth Forest Biology Conference, TAPPI. Raleigh, N.C. "The Clonal Propagation of Trees from Tissue Culture."
	August 7	First North American Forest Biology Workshop (American Society of Foresters). Michigan State University, East Lansing. "Clonal Propagation of Hardwoods from Tissue Culture."
1971	Fall	Plant Tissue Culture A-378.
1972	February 10	Introduction to Research A-197.
	April 18	National Arboretum Advisory Council, 100th Anniversary of Arbor Day, National Arboretum, Washington, D.C. Official presentation of one of the callus trees.
	April 28	Edison Elementary School, Appleton. Grades 1-6 observance of Arbor Day. Slide talk and demonstrations.

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[a] Many of these citations are listed under publications, so their titles are not given here.

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